A NEW METHOD FOR PRECIPITATING BACTERIAL EXOPOLYSACCHARIDES

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SUMMARY

Several methods for exopolysaccharide precipitation are compared and a new method is proposed which uses nitron (3,5,6-triphenyl-2,3,5,6-tetraaza[2.1.1]bicyclo-1-hexene) as the precipitating agent and gives recoveries of about 99%, using only 0.1 vol.. The highest recovery obtained with the usual methods was around 75%, using 3 vol. propanol, but this makes the final recovery more difficult.

INTRODUCTION

The increasing interest in studying microbial exopolysaccharides (EPS) is expressed by the great number of publications on this subject in the last five years. The importance of EPS lies in a wide range of possible applications. They can be used as emulsifiers, stabilisers and selective adsorbents, as well as additives for rheological control.

The main steps to obtain a bacterial polysaccharide are extraction and precipitation, the latter being followed by purification, if it is desirable. Many methods have been used to extract and to precipitate EPS from microbial cultures. A comparison of bacterial extracellular polymer extraction methods was done by Brown and Lester (1980) in order to select an extraction method suitable for routine quantitative analysis of EPS production. Concerning precipitation methods, it is possible to find in the literature a great number of different precipitating agents. The most common are organic solvents: ethanol (Moscovici et al., 1993; Otsuji et al., 1994), acetone (Kennedy and Sutherland, 1987), propanol (Williams and Wimpenny, 1977) and isopropanol (Kim et al., 1994; Fernandes et al., 1989). The quantity of the solvents is also variable: one, two or three volumes are normally used. Although, two volumes seems to be the most usual.

The aim of the present work was to select a high yield precipitation method. This was achieved by comparing the yields of the methods which have been shown to successfully precipitate extracellular polymers and by using a new method.

To account for different behaviours of neutral and acidic polysaccharides it was decided to assay commercial polysaccharides of both types and also the EPS produced by *Alcaligenes denitrificans*. This microorganism is able to produce substantial amounts of EPS having citrate as the sole organic carbon source.

MATERIALS AND METHODS

Polysaccharides: hydroxypropyl starch obtained from Växo, Sweden, was used as the neutral polysaccharide. The acidic polysaccharide was sodium alginate, obtained from Riedel-de Haën, Germany. The concentrations used were: 20 g/l and 10 g/l, respectively. The EPS produced by *Alcaligenes denitrificans* was obtained by cultivating the microorganism in citrate minimal medium: 9.28 g/l K_2HPO_4, 1.81 g/l KH_2PO_4, 1.48 g/l $C_6H_5Na_3O_7.2H_2O$ and 100 ml/l of Solution A. This solution was prepared in 1 litre of distilled water, with the following composition: 0.42 g $Na_2MoO_4.2H_2O$, 0.056 g $FeSO_4.7H_2O$, 0.0081 g $MnCl_2.2H_2O$, 0.0515 g $CaCl_2.2H_2O$ e 2 g $MgSO_4.7H_2O$. Batch cultures 3 days old were centrifuged and the supernatant was recovered to be submitted to the precipitation methods.

Precipitating agents: The precipitating agents used were: organic solvents (ethanol, acetone, propanol and isopropanol), cetyltrimethylammonium bromide (CTAB) and 3,5,6 - triphenyl-2,3,5,6 - tetraaza [2.1.1.1] bicyclo -1-hexene, commercially known as nitron.

Precipitation methods: (i) Precipitation with organic solvents - for each solvent, two precipitation assays were carried out, using 1, 2 and 3 volumes of the solvent. The solvents were kept 24 hours at -18°C, before being used. The solvent was added to the polysaccharide solution under gentle magnetic stirring and left to precipitate for 24 hours at 5°C. (ii) Precipitation with nitron - 0.1 volumes of a 10% solution of nitron in acetic acid 3% were added to the polysaccharide solution after the addition of 0.1 volumes of sulphuric acid 98%. The precipitation occurred instantaneously, however, the precipitates were allowed to stand for 24 hours, before being quantified. (iii) Precipitation with CTAB (Filali Mouhim et al. 1993) - 5 ml of sodium sulphate 0.02 M were added to 5 ml of the polysaccharide solution. 1 ml of CTAB 3% (w/v) was added by

drops to this solution. The precipitation of sodium alginate took place immediately, but it was left to stand for 24 hours.

Determination of precipitation yield: the precipitates were filtered through glass microfibre membranes (GF/C Whatman). The membranes were previously dried in a stove at 80°C during 12 h and then weighed. After filtration, they were submitted to the same drying procedure and weighed again. The quantity of precipitate was determined by subtraction of the two membrane weights.

RESULTS AND DISCUSSION

Nitron and all the organic solvents used were able to precipitate the neutral and the acidic polysaccharides. CTBA could only precipitate the acidic polysaccharide, as expected.

The yields of precipitation of hydroxypropyl starch with organic solvents are presented in Figure 1. As can be seen, propanol was the most effective precipitating agent, being followed by isopropanol, especially when three volumes were used. The increase in precipitation yield with the increase of solvent volume is a general trend for all the solvents tested.

Figure 2 shows the yields of precipitation of alginate using organic solvents. As before, propanol was the most effective. A yield of 75% could be obtained when two volumes were used.

The average yield for the precipitation of alginate with CTAB was 43%, while for hydroxypropyl starch no precipitation was detected. Although CTAB can be used selectively for acidic polysaccharides, more than 50% of alginate was not recovered.

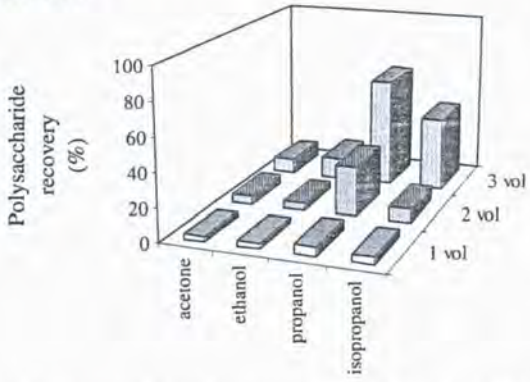

Figure 1. - % of hydroxypropyl starch recovered after precipitation using organic solvents.

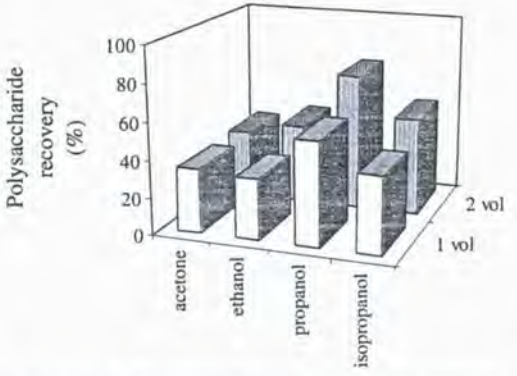

Figure 2. - % of alginate recovered after precipitation using organic solvents.

Nitron enabled the recovery of 99% of both types of polysaccharides and the process of filtration was much more easier, because the increase in volume is not very significant. When nitron is used, the recovery of the precipitate is easily achieved by centrifugation, followed by drying, minimising the losses that are difficult to avoid during the filtration process

The EPS produced by *Alcaligenes denitrificans*, was submitted to all the above mentioned precipitation methods and the highest amounts of precipitate were obtained with nitron followed by propanol and isopropanol. No precipitate was obtained with CTAB, while with nitron it was possible to obtain 0.086 g/ml.

The precipitation mechanism is still not well understood and it is important to be sure that the properties of the polysaccharide would not be affected by the precipitating agents used. To account for this possibility, after each precipitation experiment, the hydroxypropyl starch was assayed with a solution of KI. Whatever precipitating agent used, it was impossible to detect the characteristic colour of the starch/iodine reaction. So, every precipitate was dialysed against distilled water. After 48 hours of dialysis the precipitates were assayed with KI and the reaction with iodine was successful, meaning that all the precipitating agents had been removed.

For an accurate quantification (by weight) of EPS precipitated with nitron, the culture medium should be submitted to a previous dialysis process, in order to ensure the removal of salts and small molecules. Otherwise they can be precipitated as well, masking the overall quantity obtained.

CONCLUSIONS

The maximum yield of precipitation was obtained using nitron. Nitron has another advantage, it is used in small quantities (0.1 volumes), which facilitates the final recovery of the polysaccharide.

The highest yield of precipitation, using organic solvents, was obtained with 3 volumes of propanol.

CTAB is selective for acidic polysaccharides, although the precipitation yields are not very high.

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REFERENCES

Brown, M. J., Lester, J. N., (1980), Applied and Environmental Microbiology, **40**, 179-185.

Fernandes, H. L., Tomé, M. M., (1989), Biotechnology Letters, **11**, 433-436.

Filali Mouhim, R., Cornet, J. F., Fontaine, T., Fournet B. and Dubertret, G., (1993), Biotechnology Letters, **15**, 567-572.

Kennedy, A. F. D., and Sutherland, I. W., (1987), Biotechnology and Applied Biochemistry, **9**, 12-19.

Kim, S., Kong, I., Kwon, K., Rha, C., Sinskey, A. J., Kong, J., (1994), Biotechnology Letters, **16**, 789-794.

Moscovici, M., Ionescu, C., Oniscu, C., (1993), Biotechnology Letters, **15**, 1167-1172.